Spring Focus Workshop
Scientific, Regulatory and Technology Challenges in the Development of Oligonucleotide and Peptide Drugs

Just as oligonucleotides aren't peptides either…

Cecilia Arfvidsson, on behalf of the EBF

8-9 June 2023 – Malaga, Spain

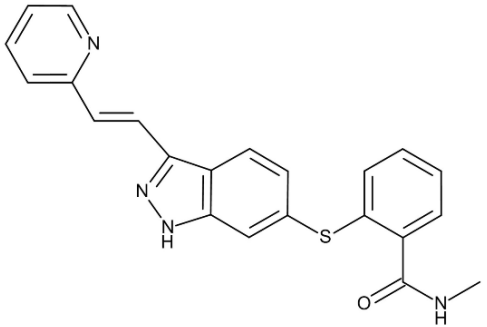
Take home messages from this goodiebag

- Oligos ≠ small molecules
- Opportunity to make the undrugable drugable
- Different modes of action
- Limited cellular uptake triggered
  - Chemical evolution and optimisation
  - Targeting strategies
- Complex bioanalytical requirements
  - ‘Novel’ analytical method may have to be explored
  - Multiple approaches often required
  - Platform strategies may be applied

Those were the times when…

Expanding pharmaceutical approaches

Small Molecules



Axitinib (Inlyta, Pfizer); $C_{22}H_{18}N_4OS$; MW = 386

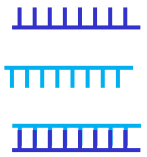
Large Molecules

Antibodies

Protein & peptides

New Modalities

Oligonucleotides and active metabolites



ASO

anti-miR

siRNA

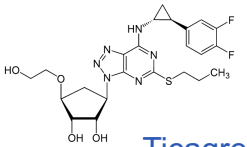
modified mRNA

mRNA

Nucleotide based therapeutics ≠ small molecules

- Size

Acetylsalicylic acid



Ticagrelor, Brilinta/Brilique

Representative 20-mer ASO

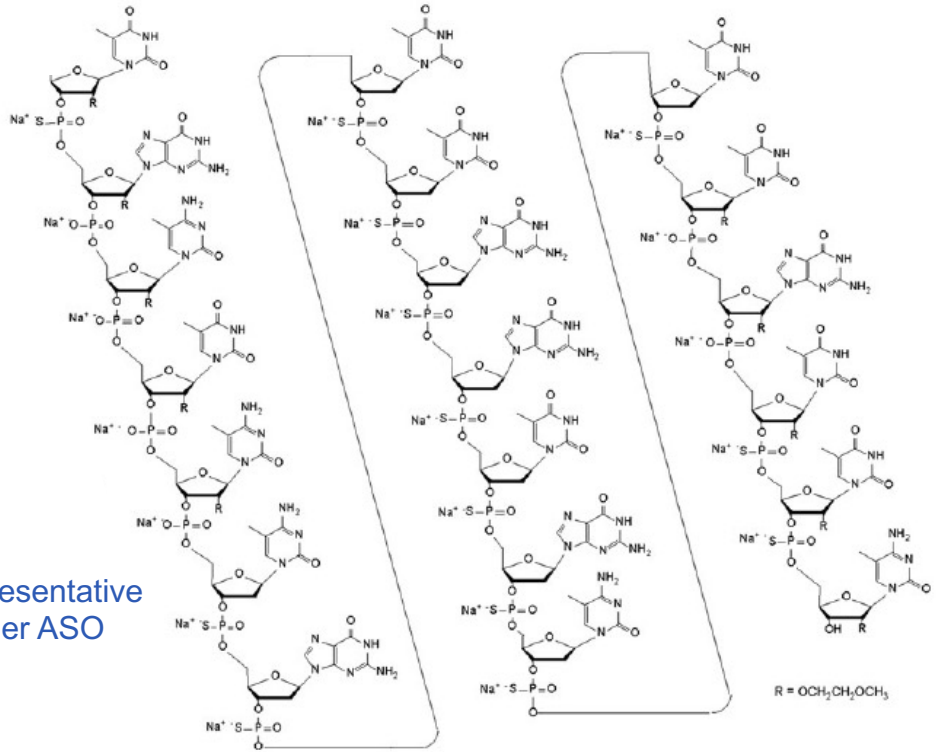


FIG. 1. Chemical Structure of ISIS 681257.

Yu et al. (2016) Nucleic Acid Therapeutics, in press DOI: 10.1089/nat.2016.0623

What are oligonucleotide drugs?

- Small pieces of modified DNA or RNA
  - Single or double stranded
  - Synthesized from chemically modified nucleotides
- Target RNA in a sequence specific manner (Watson-Crick base pairing) and intervene at genetic level
- With the aim to modulate biological pathways to cure a specific condition.
  - Knockdown of toxic protein
  - Restoration of missing protein

Evolution of RNA biology and therapeutics

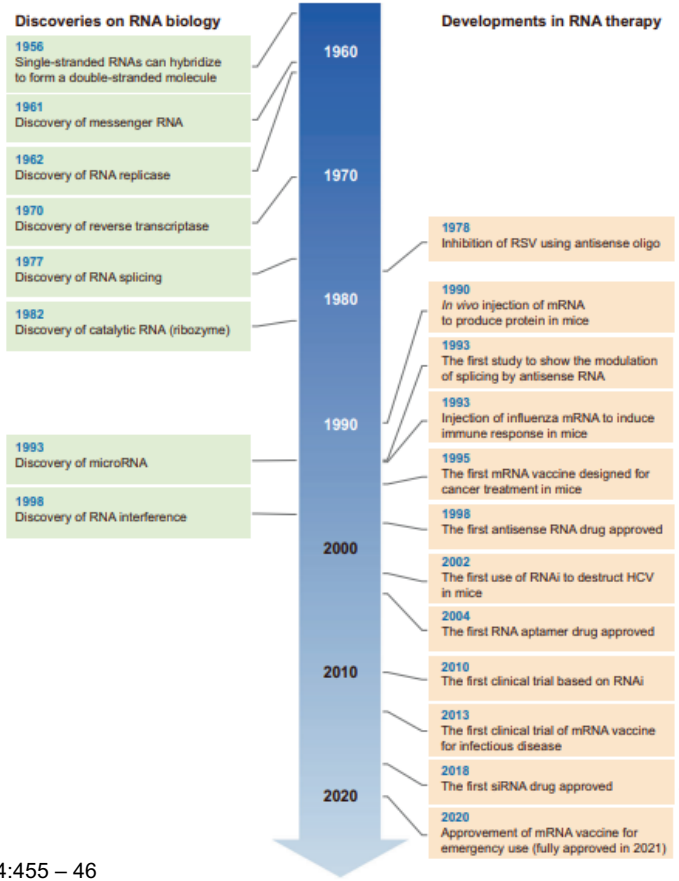
1956

First nucleic acid hybridization reactions published - RNA can form a similar configuration to DNA based on the same complementarity in its base-pairing

1993

1998

Formed the basis for the discovery of microRNA (miRNA) and RNA interference



1978

The first application of RNA base-pairing for therapeutic purposes - design of an antisense oligonucleotide (ASO) targeting the Rous sarcoma virus (RSV) to inhibit viral replication.

1998

First drug using an ASO was approved by the FDA

2018

First small interfering RNA (siRNA) drug approved

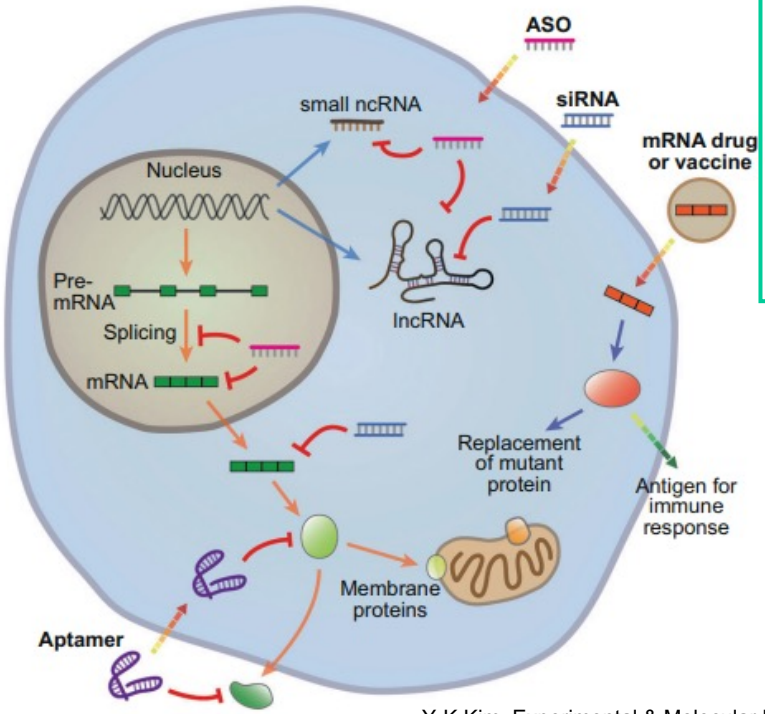
Y-K Kim, Experimental & Molecular Medicine (2022) 54:455 – 46

Nucleotide modes of action

RNA-based drugs can target various steps involved in the expression of both protein-coding and noncoding genes.

Antisense oligonucleotides (ASOs) modulate the expression of target RNAs via sequence-specific binding and can modulate splicing or induce RNase H-mediated degradation of the target mRNA

Aptamers bind to their target protein, to modulate their function.



small interfering RNAs (siRNAs) use the endogenous RNA interference (RNAi) pathway to modulate the expression of their target RNAs. Through the interaction with the Argonaute (AGO) protein siRNA produce a binding complex that recognizes its target mRNA and then induces its sequence-specific cleavage.

Exogenous mRNAs function as direct template for protein translation, either intracellular to replace or supplement endogenous protein or as antigens to elicit a targeted immune response

Y-K Kim, Experimental & Molecular Medicine (2022) 54:455 – 46

Oligonucleotide based drugs - hybridisation dependent and independent mode of action

Hybridisation dependent

- siRNA
- ASO
- Splice modifying oligos
- miR (microRNA)
- Anti-microRNA
- PGE (Precision Genome Editing)

Hybridisation independent

- Aptamers
- Immunostimulatory (CpG) oligos
- mRNA

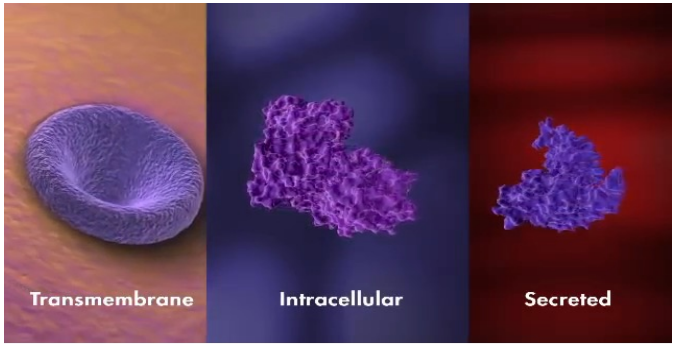
An exciting drug development area - targeting undruggable targets

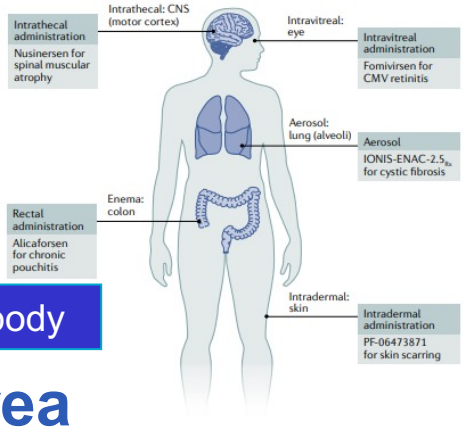
RNA Therapeutics

New way to treat diseases

Encoding a protein fine tunes the efficacious effect

Potential to target any type of protein





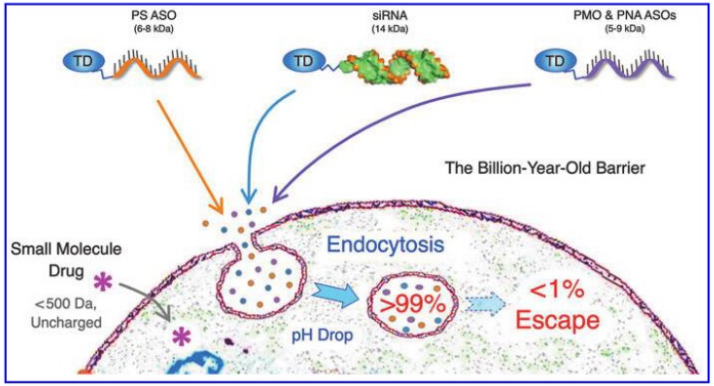
Potential to target any region of body

Potential to target any disease area

Oligonucleoride classes have different properties

- Mechanism of action
  - Intra-cellularly
  - antisense, anti-miR, siRNA, splice-correction, aptamer, miR mimic etc
- Oligo design
  - Single or double stranded, length, gapmer or non-gapmer, phosphate overhangs etc.
- Chemistry
  - Charged phosphorothioate backbone or not; 2’ ribose modifications: O-ME, MOE, LNA, cEt, 2’F; conjugations (e.g. GalNAc)
- Administration vehicle
  - Lipid formulation or saline
- Exposure – often short in circulation due to endosome uptake, often long in target tissue

Example on ASO/siRNA delivery to target cell



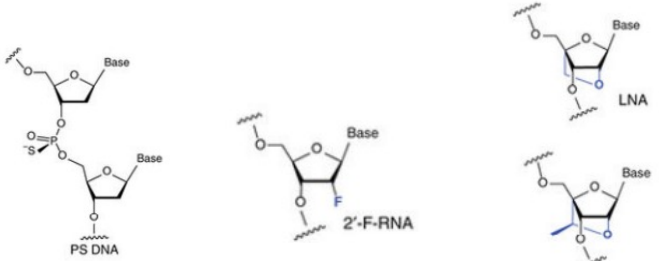
Delivery of RNA Therapeutics: The Great Endosomal Escape!
Nucleic Acid TherapeuticsVol. 32, No. 5 Review 2022

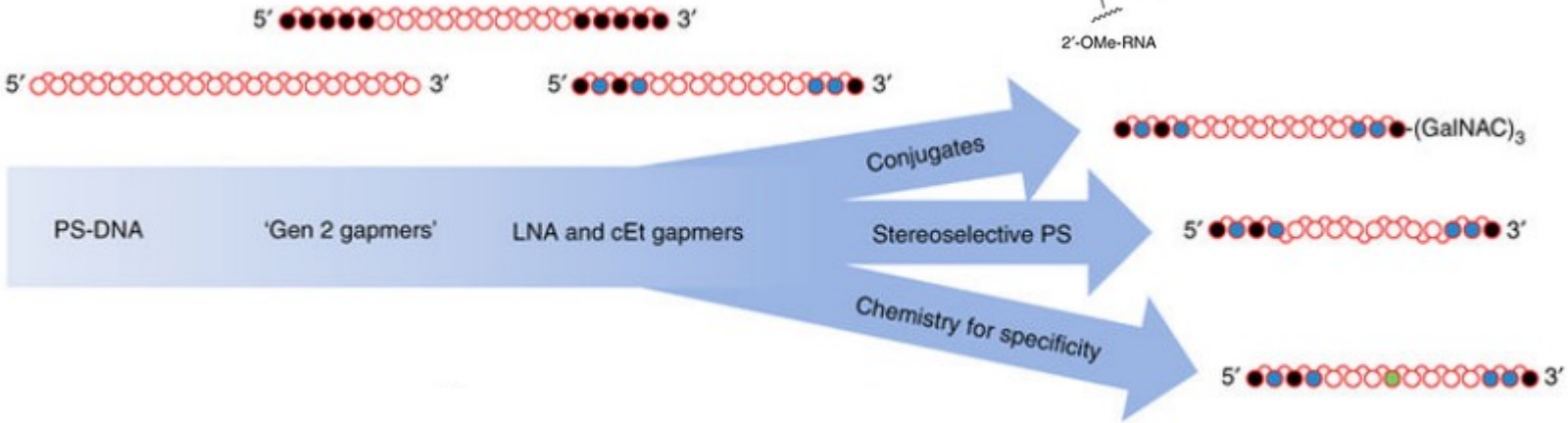
Direct comparisons can be made within a class only

Evolution of ASO technology

Key steps for ASO technology maturation:

- Phosphorothioate backbone
- Gapmer design with MOE 2’ ribose modifications
- LNA and cEt bridged 2’ ribose modifications
- GalNAc conjugates

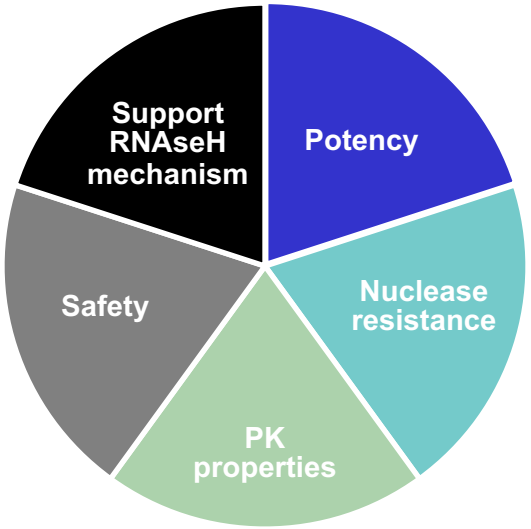


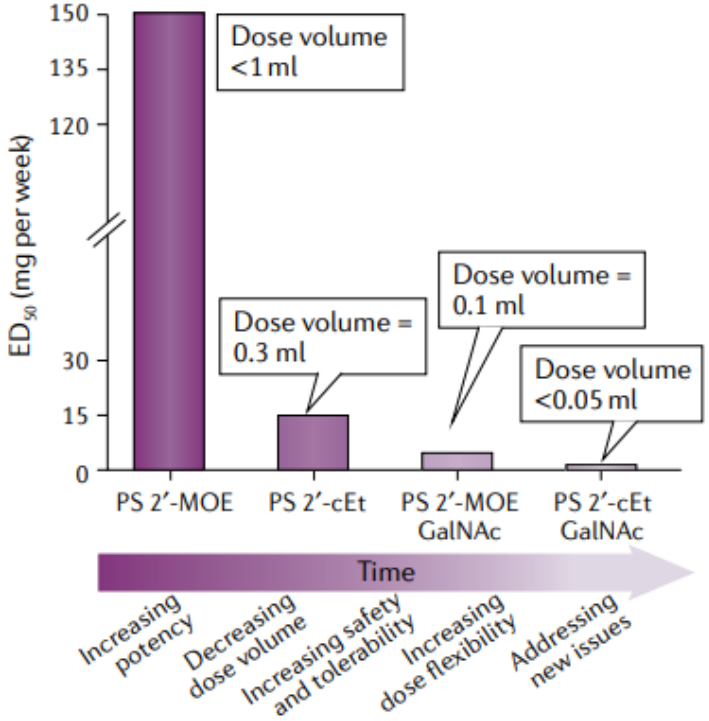


Khvorova, A., and Watts, J.K. (2017). Nat Biotechnol *35*, 238-248.

ASO chemistry optimisation

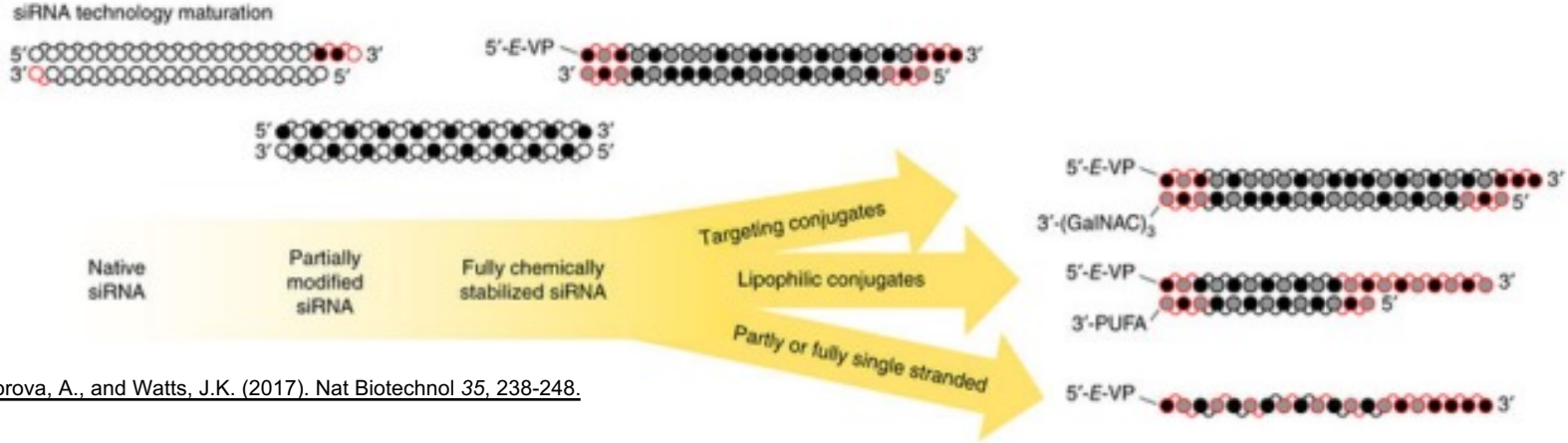
- Balance of several properties





S.T Crooke et al. Nature Reviews, volume 20 (2021)

Evolution of siRNA technology



Khvorova, A., and Watts, J.K. (2017). Nat Biotechnol *35*, 238-248.

Key steps for siRNA technology maturation

- Minor stabilizing modifications delivered in LNP
- Extensive modifications (2'F, O-ME, PS backbone)
- Stabilized 5'Phosphate group 5'E-VP
- GalNAc conjugates allowed moving away from LNP to saline injections only

How to overcome a billion years of evolutionary defenses that have kept RNAs on the outside of cells from invading the inside of cells?

The lipid bilayer was fundamental in creating life and in protecting it from invading RNAs…

- allowing small neutral, slightly hydrophobic molecules <1,000 Da to passively diffuse across
- preventing large, charged molecules, like RNAs, from crossing

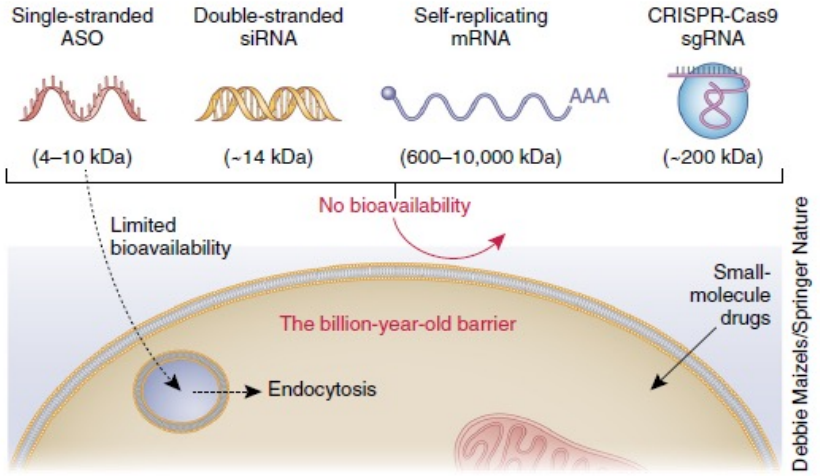
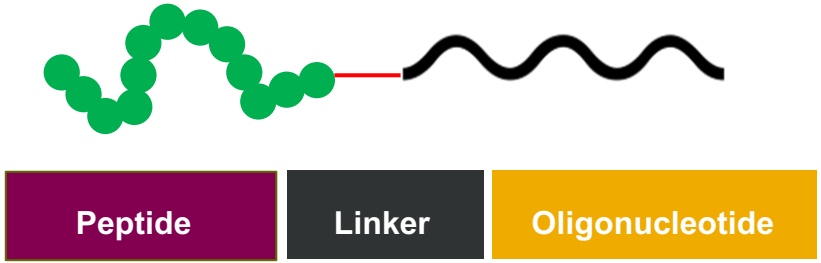
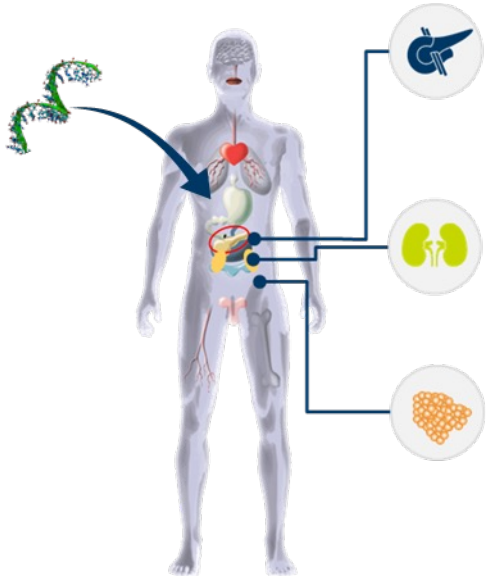


Figure 1 The four-billion-year-old lipid bilayer protects cells from invading RNAs. Unlike small-molecule drugs that can slip across the lipid bilayer, with the exception of some single-stranded phosphorothioate ASOs that can productively enter cells, the vast majority of RNA-based therapeutics are too charged and/or too large to enter cells, and require a delivery agent.

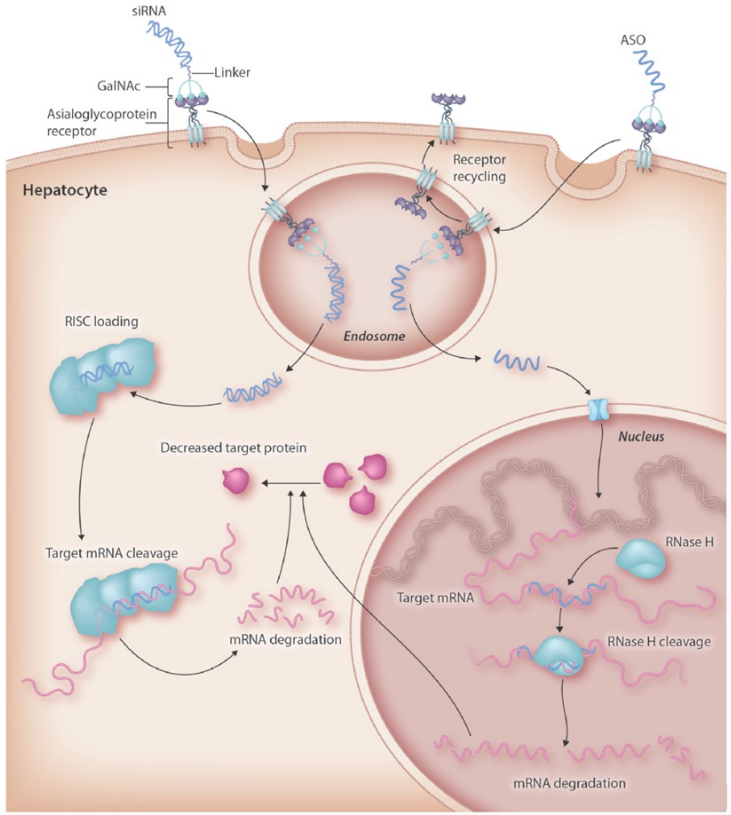
Targeting strategies for ASOs and siRNA

- Increase drug efficacy/potency
- Minimise off target effects
- Target specific receptors using ligands enhancing functional uptake



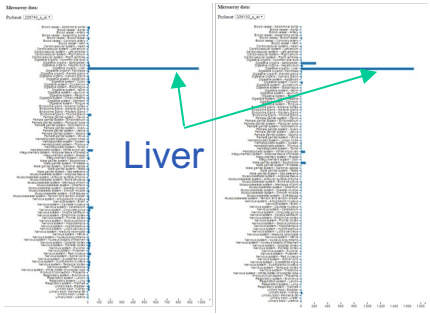


Liver targeted delivery of GalNAc conjugated siRNA & ASO



- Liver hepatocytes express the asialoglycoprotein receptor (ASGPR)
- GalNAc binds to the ASGPR and is taken up in endosomes
- In endosomes the conjugate dissociates from the receptor and GalNAc sugars and branches are lysed
- The oligonucleotide escapes into the cytoplasm (poorly understood mechanism)

Human mRNA expression

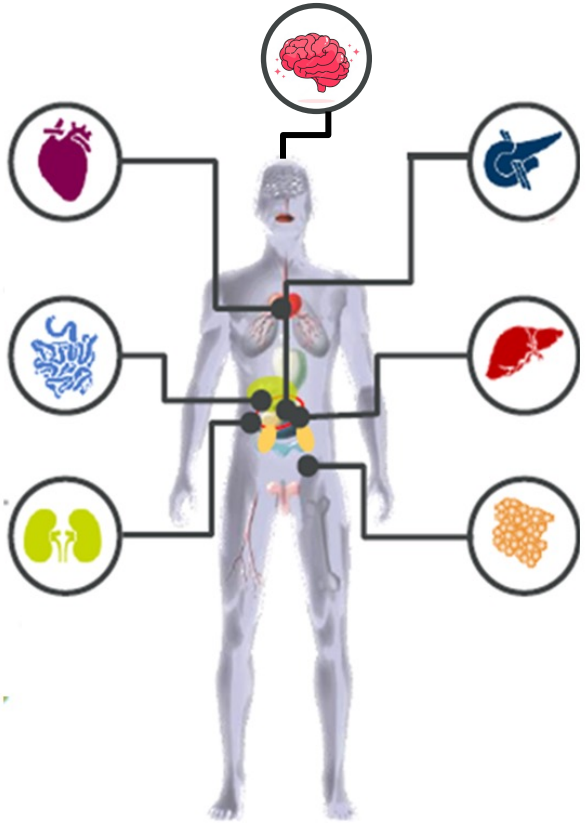


Debacker et al Molecular Therapy Vol. 28 No 8 August 2020

Targeting beyond the liver

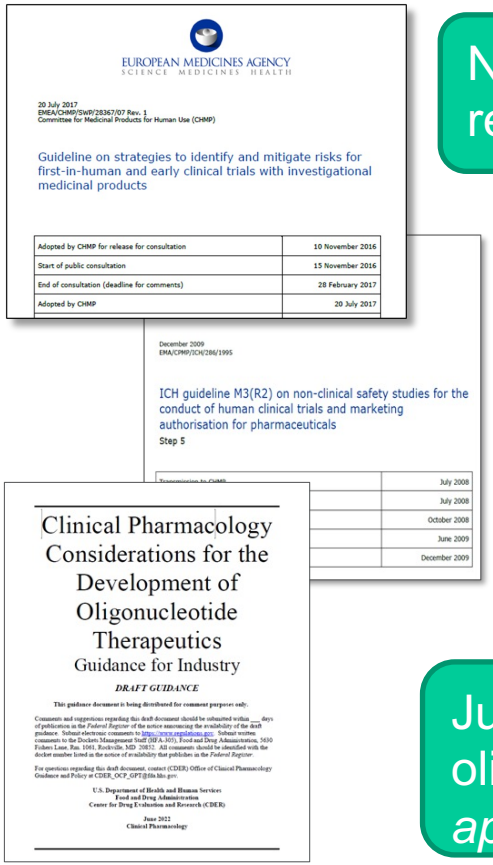
Hydrophobic modification of siRNAs with fatty acids or cholesterol has been explored as a delivery strategy

Kidney will likely be the next tissue clinically targetable by systemically delivered RNAi.



Conjugate-mediated delivery of oligonucleotides to non-primary tissues, including **heart, pancreas, lung, and tumor** will require further advances in chemistry to take advantage of mechanisms driving oligonucleotide clearance, tissue distribution, cellular uptake and endosomal escape.

Regulatory landscape



NON-CLINICAL PK package: ASOs are regarded as small molecules

From ICH M3: "*For products using innovative therapeutic modalities (e.g., siRNA), as well as vaccine adjuvants, particular studies can be abbreviated, deferred, omitted, or added.*"

Strategies and justifications need to be confirmed at interactions with the Health Authorities

June 2022; Draft FDA guidance for the development of oligonucleotides: "*Characterize relevant metabolites using appropriate analytical methods*"

Complex bioanalytical requirements

Biodistribution / modelling	• Does the oligo reach the intended site of action? • What happens to the targeting agent?
Biotransformation	• What metabolites are formed? • How do they contribute to overall activity and safety?
Half Life	• *In vivo* stability of the linker? • Analytical methods are not always mutually compatible
Immunogenicity	• Specificity to oligo or targeting agent? • How does this impact uptake to target tissue?

Session 6 '*Do metabolite quantification strategies need to change*'

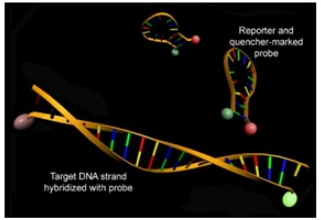
Session 3/5 '*More than one way to skin a cat*'

Session 4 '*Immunogenicity …*'

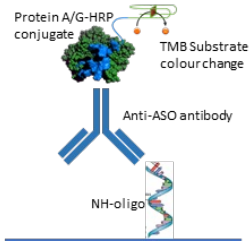
Session 2 '*Preparing for success = preparing early*'

Different analytical methods and approaches have to be explored

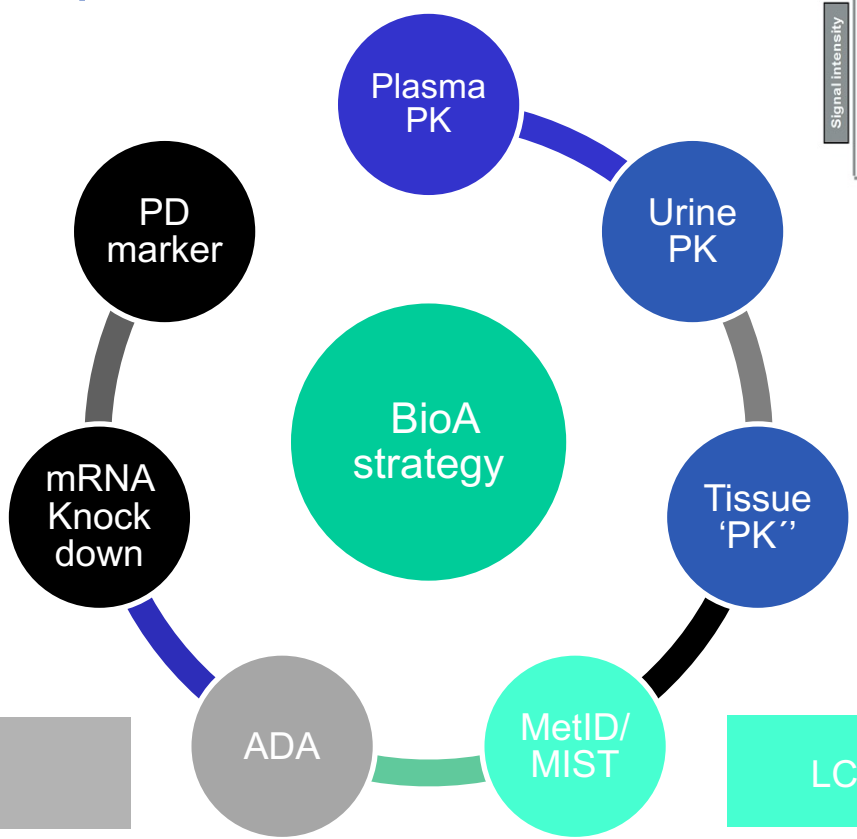
Complex BioA strategy with multiple assays and platforms

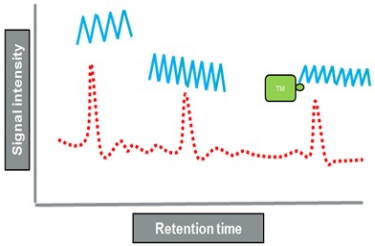


PCR

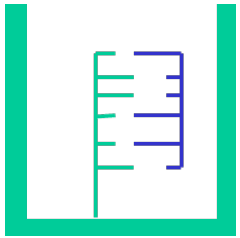


ELISA





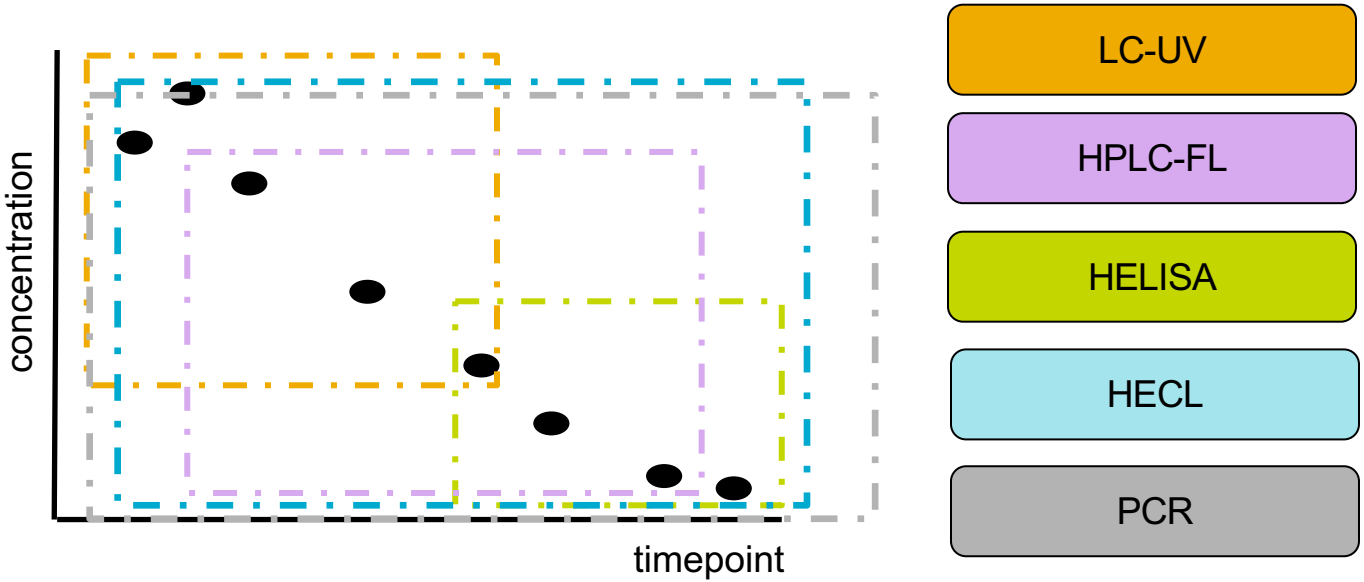
LC-MS/MS, Hybridisation Assay (ELISA, ECL LC-FI, Gyros), PCR



LC-MS or LC/UV

PK assay considerations - sensitivity vs. specificity/selectivity

- Oligonucleotides accumulate in tissues (to high concentrations)
- At later timepoints and for quantification in plasma it is often necessary to use more sensitive methods (HPLC-FL or HELISA/HECL)



Multiple bioanalytical approaches often required

For further consideration

- Multiple approaches are often applied for oligonucleotides
  - What value may the BioA scientist add to the cross functional team discussions to ensure that the data is used in an optimal as well as accurate way?

- The need for complex bioanalytical solutions to support oligonucleotides in combination with unclear regulatory requirements may easily trigger an uncertainty to what is needed and results in that we do too much …
  - Should we always measure just because we can?
  - What are the BioA lab's responsibilities and possible role to challenge what endpoints that are assessed?
  - What platform strategies and historical data may be considered?

Questions

Acknowledgements

- EBF OC
- EBF community

Contact Information

Questions: info@e-b-f.eu

European Bioanalysis Forum vzw

www.e-b-f.eu